The origin and evolution of social insect queen pheromones: Novel hypotheses and outstanding problems

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Queen pheromones, which signal the presence of a fertile queen and induce daughter workers to remain sterile, are considered to play a key role in regulating the reproductive division of labor of insect societies. Although queen pheromones were long thought to be highly taxon-specific, recent studies have shown that structurally related long-chain hydrocarbons act as conserved queen signals across several independently evolved lineages of social insects. These results imply that social insect queen pheromones are very ancient and likely derived from an ancestral signalling system that was already present in their common solitary ancestors. Based on these new insights, we here review the literature and speculate on what signal precursors social insect queen pheromones may have evolved from. Furthermore, we provide compelling evidence that these pheromones should best be seen as honest signals of fertility as opposed to suppressive agents that chemically sterilize the workers against their own best interests.

Keywords:
cuticular hydrocarbons; fertility signals; queen pheromones; reproductive conflict; reproductive division of labour; social evolution; social Hymenoptera

Introduction

A eusocial lifestyle, characterized by a reproductive division of labor between fertile queens and largely sterile workers, has evolved independently at least 22 times within the animal kingdom and nine times amongst Hymenoptera, including ants and several groups of bees and wasps [1]. The evolutionary causes of this advanced form of cooperation are a long-running, contentious topic of debate [2, 3]. Mechanistically, pheromones emitted by the queen have long been thought to play a key role in suppressing worker reproduction [4]. Yet, the origin and evolution, as well as the identity, of such pheromone signals have long remained elusive. In fact, decades of research have resulted in successful identification of only a few, structurally unrelated, sterility-inducing queen pheromones in intensively studied model species, such as the domestic honeybee [4–7] and *Reticulitermes* termites [8]. In the honeybee, for example, it was shown that queen mandibular pheromones, containing a blend of volatile carboxylic acids (9-ODA, 9-HDA) and aromatics (HOB, HVA), are used both as female sex pheromones, to attract males during their mating flight, as well as to induce sterility in the offspring workers [4–7] (Fig. 1A, B). As there was no evidence, however, that either these or related substances function as queen pheromones outside honeybees, it was long thought that queen pheromones act in a highly taxon-specific manner [4]. This theory was overturned by recent studies that

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Abbreviations:
3-MeC$_{27}$, 3-methylheptacosane; **3-MeC$_{29}$,** 3-methylnonacosane; **3-MeC$_{31}$,** 3-methylhentriacontane; **9-HAD,** (+) and (−)-9-hydroxy dec-2-enoic acid; **9-ODA,** (*E*)-9-oxo-2-decenoic acid; **10-HAD,** 10-hydroxy-2-decenoic acid; **10-HDAA,** 10-hydroxydecanoic acid; **C$_{29:1}$,** (*Z*)-9-nanocosene; **CA,** coniferyl alcohol; **E1-E4,** eicosyl, docosyl, tetracosyl and hexacosyl oleate esters; **HOB,** methyl-*p*-hydroxybenzoate; **HVA,** homovanillyl alcohol; **MO,** methyl octadec-9-enoate; ***n*-C$_{25}$,** pentacosane; ***n*-C$_{27}$,** heptacosane; ***n*-C$_{28}$,** octacosane; ***n*-C$_{29}$,** nonacosane; ***n*-C$_{31}$,** hentriacontane; **ODPs,** oviposition deterring pheromones; **PA,** palmityl acohol; **QMP,** queen mandibular pheromones.

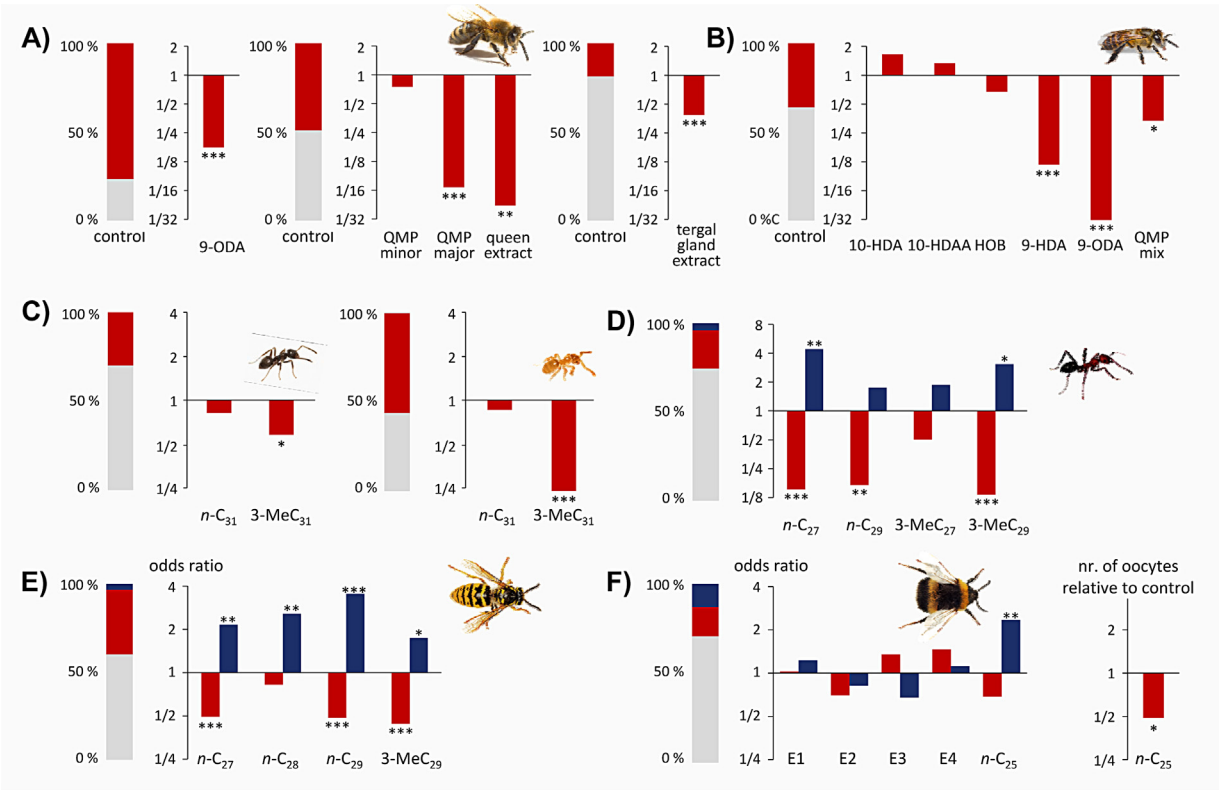

Figure 1. Bioassays demonstrating the chemical identity of sterility-inducing queen signals in social Hymenoptera. Bioassays with different queen-characteristic compounds were used to test the effect on worker ovary development (red) or worker ovary regression (blue) relative to a queenless control (stacked bars on the left) in different social insect species (**A:** domestic honeybee *Apis mellifera*, **B:** Asian honeybee *Apis cerana*, **C:** black garden and yellow meadow ant *Lasius niger* and *L. flavus*, **D:** Iberian ant *Cataglyphis iberica*, **E:** common wasp *Vespula vulgaris*, **F**: buff-tailed bumblebee *Bombus terrestris*). In honeybees, queen mandibular pheromones [6, 21, 22] as well as tergal cuticular compounds [52] are active as queen-pheromones, whereas in ants, the common wasp and bumblebees, specific queen-characteristic linear alkanes and/or 3-methyl branched alkanes were shown to act as conserved queen pheromone signals [9–12]. By contrast, the control hydrocarbon *n*-C_{31}, which was not specific to the queen caste, had no effect in *Lasius* ants [9, 10] and four queen-characteristic esters (E1–E4) also had no effect on worker reproduction when tested individually in the bumblebee [11]. Abbreviations: QMP major = 9-ODA, 9-HDA, HOB, and HVA, QMP minor = MO, CA and PA. Significance levels were calculated using binomial linear models or binomial linear mixed models (for proportions of workers with active ovaries) or Poisson mixed models (for the mean number of developing oocytes in worker ovaries in the treated groups vs. in the control in panel F) and are indicated as follows: * < 0.05, ** < 0.01, *** < 0.001.

characterized queen pheromones from a wider set of species [9–12]. In these studies, bioassays were used to demonstrate that specific, structurally simple long-chain hydrocarbons, present on the queen's cuticle, were used as sterility–inducing queen signals across several widely divergent species of social insects, including three species of ants [9–11], a bumblebee [11, 12] and the common wasp [11] (Figs. 1 and 2). This was surprising, given that these groups each independently evolved eusociality (Fig. 2), and implies that social insect queen pheromones must be very ancient and are likely derived from a signalling system that was already present in their common solitary ancestors [11, 13].

The aim of this perspective article is to take these recent findings as a starting point to propose several hypotheses about the evolution of social insect queen pheromones from prototypical, ancestral cues, or signals in solitary or primitively eusocial ancestors. This focus on the origin of social insect queen pheromones is distinct from some other reviews in the field, which have mainly dealt with the evolutionary stability of queen pheromones [14–16] or the physiology of the queen's inhibitory effect [4]. Towards the end of our review, however, we will also come back to some outstanding controversies, such as whether queen pheromones should best be interpreted as "honest signals" for the presence of a healthy, fertile queen or as suppressive agents that chemically sterilize workers against their own reproductive interests [17–19]. We argue that the evidence is in favor of the queen signal hypothesis. Lastly, we suggest some interesting avenues for future research.

Queen pheromones in bees and termites have volatile components

Although queen pheromones have long been known to play a key role in regulating insect sociality, progress in characterizing their chemical nature and identifying their site of production has been slow. In the honeybee, much early work was concentrated on the queen mandibular pheromone (QMP), which was found to contain a blend of volatile

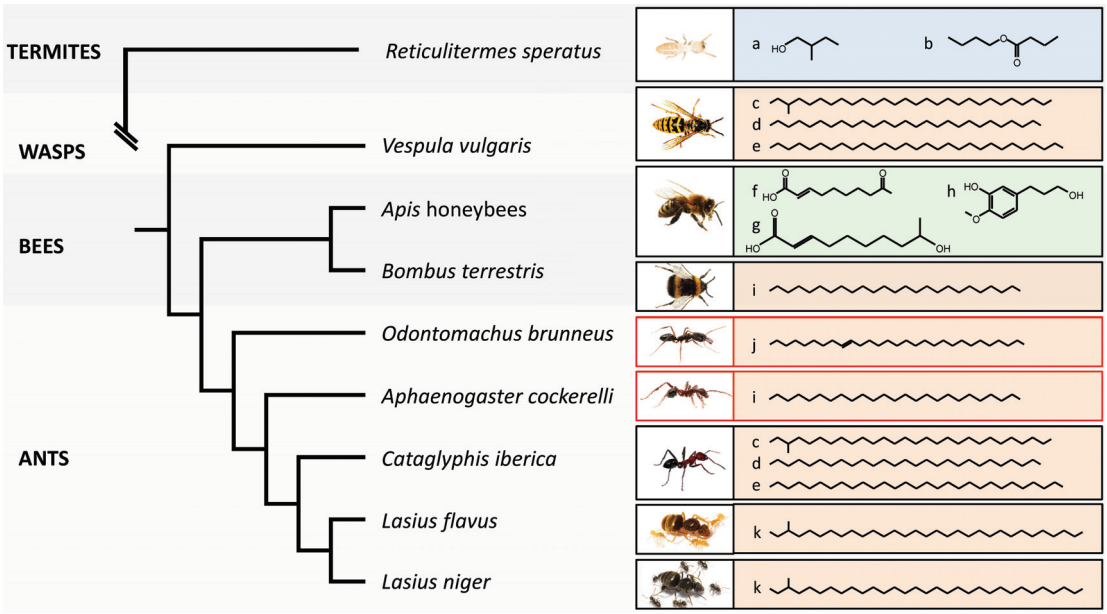

Figure 2. Evolutionary history and identity of sterility-inducing queen and fertility signals in social insects. In ants, bumblebees, and Vespine wasps, which each independently evolved eusociality (alternately shaded areas), cuticular hydrocarbons (peach) appear to act as conserved sterility-inducing queen pheromones [9–11]. By contrast, in the honeybee (light green) and termites (light blue), other volatile compounds, such as keto acids, esters, and alcohols appear to be used as queen pheromones [6–8], even though non-volatile cuticular compounds are also likely important [37, 51, 52, 54]. The boxes show the chemical structures of the compounds that have been shown to be active in bioassays (*R. speratus*: a = *n*-butyl-*n*-butyrate, *b* = 2-methyl-1-butanol, *V. vulgaris*, and *C. iberica*: *c* = 3-MeC$_{29}$, *d* = *n*-C$_{27}$, *e* = *n*-C$_{29}$, *A. mellifera*: QMP pheromones [98], *f* = 9-ODA, *g* = 9-HDA, *h* = HVA, *B. terrestris*, and *A. cockerelli*: *i* = *n*-C$_{25}$, *O. brunneus*: *j* = C$_{29:1}$; *L. flavus* and *L. niger*: *k* = 3-MeC$_{31}$). In all these species, bioassays have demonstrated a direct inhibitory effect on reproduction. In the termite species this occurs by inhibiting the differentiation of new reproductives [8]. In the other species, queen signals inhibit the worker's reproduction [6, 9–11], whereas two experimentally demonstrated fertility signals (red boxes) are characteristic of both queens and reproductive workers, and are used as cues to aggressively police egg-laying workers [40, 41]. In the common wasp, 3-MeC$_{29}$ is also used as a queen egg-marking signal that helps workers to recognize queen-laid eggs from worker-laid eggs and selectively destroy or "police" worker-laid eggs [42]. Modified after Van Oystaeyen et al. (2014) [11].

carboxylic acids (9-ODA, 9-HDA) and aromatics (HOB, HVA) as well as some other minor compounds [20]. These pheromones have been shown to have multiple effects, including attraction of drones during the queen's mating flight, inhibition of queen rearing, induction of queen retinue behavior (i.e. attract workers around the queen), and induction of sterility in the offspring workers [4, 7, 21–23]. With respect to the inhibition of worker ovary development in honeybees, particularly the two major QMP compounds 9-ODA and 9-HDA appeared to be bioactive [6, 21, 22]. This was shown by the fact that in the domestic honeybee *A. mellifera*, a mix of the major QMP pheromones was almost as active as a complete queen extract (Fig. 1A, [6]). In addition, when tested individually, 9-ODA and both 9-ODA and 9-HDA induced strong worker ovary inhibition in *A. mellifera* (Fig. 1A, [21]) and the Asian honeybee *A. cerana* (Fig. 1B, [22]), whereas other, minor QMP compounds, such as MO, CA, and PA or 10-HDA, 10-HDAA, and HOB, did not have an effect in *A. mellifera* and *A. cerana* (Fig. 1A, B, [6, 22]). Recently, the enzymes involved in the production of the honeybee QMP blend were described [24, 25], and the olfactory receptor *AmOR11* was identified as the putative receptor for 9-ODA [26]. Esters produced in the Dufour's gland from queen's have also been found to be attractive to workers, but did not significantly affect worker ovary development [23, 27, 28]. Finally, the brood pheromones ethyl palmitate and methyl linolenate and the brood volatile *E*-β-ocimene also inhibit worker ovary development [23, 29–31], presumably to stimulate workers to care for the brood and, if possible, rear young female larvae into replacement queens. Recent chemical analyses have demonstrated that small amounts of ethyl palmitate and β-ocimene are also released by the queen herself, suggesting they are also part of the queen pheromone blend [4, 23, 30]. In the bumblebee *Bombus terrestris*, studies had originally suggested that, similar to the honeybee, volatiles produced by the mandibular gland act as queen pheromones [32]. Nevertheless, this was contested by more recent studies, which instead hinted at the importance of nonvolatile compounds ([11, 33–35], reviewed in [36]). Finally, in the termite *Reticulitermes speratus*, some volatile compounds, including an alcohol and an ester, were shown to inhibit reproduction of temporary helpers [8].

Non-volatile hydrocarbons act as conserved social insect queen pheromones

Although the evidence cited above suggests that various structurally variable volatile queen pheromones may contribute to the inhibitory effect of the queen on workers, bioassays

have shown that non-volatiles may in fact be of relatively greater importance in most social insect groups, including in wasps, ants, and bumblebees [11, 37]. Recently, these non-volatile queen pheromones were suggested to be specific fertility-linked long-chain hydrocarbons, present on queen' s cuticle [9–12, 15, 38, 39]. For ants, this theory was supported by a set of experiments by Holman and co-workers, in which it was shown that in the black garden and yellow meadow ants, the queen-characteristic hydrocarbon 3-methyl hentriacontane (3-MeC_{31}) induced strong ovary inhibition when administered to queenless worker groups, whereas the control non-queen-specific hydrocarbon hentriacontane (n-C_{31}), which was not specific to queen caste, had no effect (Fig. 1C, [9, 10]). Subsequently, other studies by Holman [12] and Van Oystaeyen et al. [11] generalized this finding by showing that queen-characteristic hydrocarbons also inhibited worker ovary development or caused secondary ovary regression in three other species belonging to three independently evolved social insect lineages, namely in the Iberian ant (Fig. 1D), the common wasp (Fig. 1E), and the buff-tailed bumblebee (Fig. 1F). The importance of hydrocarbons was also highlighted by the fact that in the bumblebee, four queen-characteristic esters (E1–E4) had no effect on worker reproduction when tested individually, whereas the queen-characteristic linear alkane pentacosane caused secondary ovary regression [11] and reduced the average number of developing oocytes present in workers' ovaries [12]. This confirmed previous studies that indicated the importance of non-volatile cuticular compounds, as opposed to volatile QMPs, as queen pheromones in the bumblebee [33–36]. Furthermore, several studies have shown that particular fertility-linked hydrocarbons are used by workers to stop other workers from reproducing [40–42]. In particular, in two ants species, specific fertility-linked cuticular hydrocarbons were shown to enable workers to recognize and aggress other egg-laying workers ([40, 41], Fig. 2), whereas in the common wasp, one of the main sterility-inducing hydrocarbon queen pheromones was shown to also be used by the queen to mark her eggs [42], thereby facilitating workers to selectively cannibalize or "police" worker-laid eggs [43].

The importance of hydrocarbons as queen pheromones or fertility cues demonstrated in these experimental bioassays has also further been supported by detailed phylogenetic analyses [10, 11], with ancestral state reconstruction showing that saturated hydrocarbons were the most common chemical class of fertility-linked cues across more than 60 species of ants, bees, and wasps ([11], Fig. 2). In addition, queen and fertility signals show a large degree of evolutionary conservation [10, 11]. Queen-characteristic 3-methyl-branched hydrocarbons, for instance, were found to be bioactive across different species of *Lasius* ants [10], and identical or structurally related 3-methyl-branched hydrocarbons and long odd-chain linear alkanes were found to be queen-specific across a great number of other social insect species [11]. The exact stereoisomers of the monomethyl branched alkanes used as queen pheromones have not been established, but a recent study has suggested that it is most likely the R form [44]. In the future, conservation should still be confirmed at the biosynthetic or receptor level as well, even though for long chain hydrocarbons, only limited knowledge is available with respect to the exact enzymes that are used to generate the large diversity of compounds produced [45] and the exact receptors that are used to perceive them [46–48].

Finally, tentative evidence for cuticular compounds being used as queen pheromones also exists for honeybees, stingless bees and termites. In the honeybee, queens from which the mandibular gland was removed still inhibited worker reproduction [49, 50] and extracts of the tergal glands, which produce specific compounds on the dorsal part of queen's cuticle [23, 51], also partially inhibited worker ovary development (Fig. 1A, [52]). Specific bioactive cuticular compounds have as yet not been identified, but either unsaturated hydrocarbons [51] or specific esters or fatty acids [23] are amongst the possibilities. In addition, in the stingless bee *Friesella schrottkyi*, it has recently been shown that non-polar cuticular queen extracts inhibit worker reproduction, and several linear alkanes, including pentacosane, as well as some monomethyl branched alkanes, induced an electroantennographic response in workers [53]. Lastly, in termites, non-volatile hydrocarbons, specific for functional reproductives, have been suggested to be part of the bioactive queen pheromone blend in several species [37]. In fact, in the termite *Cryptotermes secundus*, experimental alteration of reproductive hydrocarbon profiles induced by the downregulation of a gene involved in hydrocarbon biosynthesis has been shown to result in the loss of the queen's inhibitory effect [54].

How do insects balance the different signalling functions of cuticular hydrocarbons?

The new found function of cuticular hydrocarbons (CHCs) as conserved social insect queen pheromones is surprising, given that they are ubiquitous compounds and have a very simple chemical structure. The primary function of CHCs in insects is to prevent water loss and provide a protective, waxy coating of the insects' exoskeleton [55]. Nevertheless, variation in the position of methyl branches or double bonds locations allows for a great number of possible hydrocarbon structures, which makes them also very suitable for chemical signalling purposes [55–59]. In solitary insects, for example, CHCs may play a role in species recognition and identification of mating partners [56, 60], and in social insects, they have acquired a role in the recognition of nestmates [61], species [55], sex [62], caste, and reproductive status [9–11, 38, 39, 63]. These diverse functions imply that a role as a queen pheromone has to balance with other communicative functions and that any caste-specific signal would have to dominate other environmental sources of CHC profile variation [56]. For example, the fact that CHCs communicate both colonial origin (nestmate recognition) and reproductive caste (fertility signalling) could cause a possible conflict, as a role in nestmate recognition would require colonial uniformity, whereas caste specificity necessitates different compositions for different members of the colony [46]. There are several ways in which these contrasting requirements could be reconciled. One is through the use of distinct subsets of hydrocarbons for each function, possibly using two

separate perceptory or neural processing systems [46]. In support of this theory, empirical evidence suggests that different sets of hydrocarbons are used for both functions [11, 61]. Alternatively, it could be that the perceptory system integrates different functions in a context-specific hierarchical fashion [4, 46]. Recently, suggestive evidence for a hierarchical model of perception has been collected in *Odontomachus* ants, where recognition of nestmate signals was found to precede caste and task-specific recognition [64]. The exact neural pathways that are used to effectively filter out colony- and caste-specific signals, however, remain controversial. In ants, chemosensory sensilla on the antennae have been implicated in the perception of hydrocarbons used in nestmate recognition [46, 47], and appear to filter incoming signals by responding only to exposure to non-nestmate hydrocarbon profiles [47]. Further signal integration and processing, however, also likely occurs in the antennal lobes and higher brain centers [46]. Finally, in the fruit fly, gustatory receptors in the mouth parts and legs, whose inputs are integrated in the suboesophagial ganglion, are also involved in the perception of long-chain hydrocarbons [48].

Queen pheromones have antecedents in solitary ancestors

The notion that identical or structurally similar queen pheromones are used across different groups of social insects suggests that queen pheromones were co-opted from cues or signalling systems that were already present in their common solitary ancestors [11, 13]. This raises the question of what these ancestral, prototypical signalling systems could have looked like. Below we offer several hypotheses as to how ancestral hydrocarbon cues or signals in solitary ancestors could later have evolved into social insect queen pheromones. These hypotheses are based on the known functions and biosynthesis patterns of hydrocarbons in solitary species.

Queen pheromones may be derived from by-products of ovary development

One of the most popular hypothesis that could explain queen pheromone signal conservation is that such pheromones could be derived from fertility cues produced as by-products of ovarian development [39, 65, 66]. This is supported by the fact that ovarian activation causes distinct changes in the cuticular hydrocarbon profiles in social insects [11, 39, 63, 65] and many solitary arthropods (Table 1, reviewed in [55]), and that some of these fertility-linked hydrocarbons are similar in structure to known hydrocarbon queen pheromones [9–11]. Furthermore, intrinsic physiological links between odour and reproductive status have been shown by the fact that the production of fertility-linked hydrocarbons is under direct endocrine control in both solitary insects and Polistinae wasps (Table 1, reviewed in [55]). Lastly, in both ants [40, 41] and honeybees [67], reproductive workers produce specific compounds, including both hydrocarbons [40, 41] and Dufour's gland compounds [68, 69], which enable other workers to recognize and aggress them in order to stop them from reproducing inside the colony [40, 41, 67]. This suggests that odour and fertility are somehow intrinsically linked and that they represent so-called "indices of fertility" [14, 66], which cannot be easily faked [70]. Along with the evolution of a reproductive division of labor, by-products of fertility could have evolved into recognition cues and subsequently into dedicated queen signals, while preserving the characteristic chemical structure of such fertility-linked compounds in the original ancestors.

Queen pheromones may be derived from sex pheromones

An alternative hypothesis is that queen pheromones are derived from compounds that were originally used in courtship or mate recognition in solitary ancestors. This is plausible, given that female sex pheromones generally start to be produced around the time of mating, just before egg-laying commences [62], implying that pheromone production would only have to be extended until the emergence of the first daughters to enable a sex pheromone to evolve into a queen pheromone. On the other hand, unlike queen pheromone signals, female sex pheromone signals are directed at males rather than females, and the signal does not inhibit reproduction, so under this scenario, these effects would still have needed to evolve secondarily. Support for an origin as a female contact sex pheromone comes from the fact that compounds similar in structure to known hydrocarbon queen pheromones or fertility-signalling pheromones [9–11, 40, 41] are used in mate recognition or as contact sex pheromones in many solitary insects (e.g. [71]), and that there is also evidence that cuticular hydrocarbons act as contact sex pheromones in some ants and primitively eusocial Halictid bees [62], even if a joint function as a queen pheromone has not been demonstrated (Table 1). In addition, honeybee QMP serves a dual role as a sex pheromone and sterility-inducing queen signal [4], suggesting that the latter function might have evolved secondarily. As yet, it remains unknown, however, whether any of the characterized hydrocarbon queen pheromones [9–11] also double up as contact sex pheromones.

Queen pheromones may be derived from oviposition deterring pheromones

A third hypothesis that has been suggested is that social insect queen pheromones might be derived from so-called oviposition deterring pheromones [13, 72], which are used to regulate the reproduction among adult females of solitary insects. In support of this hypothesis, females of many solitary insects reduce their egg-laying in response to pheromonal cues ("oviposition deterring pheromones" or ODPs) left by conspecifics [72], and such ODPs reduce the egg-laying of other females [72], as is the case for social insect queen pheromones. Furthermore, the ODPs of the parasitoid wasp *Eupelmus vuilleti* include several long-chain linear alkanes and 3-methyl-branched alkanes [73] that are identical or structurally similar to known queen pheromones [9–11].

Table 1. Non-mutually exclusive hypotheses about the origin of social insects queen pheromones

Hypotheses and derived predictions	Support pro or contra
1. Queen pheromones evolved from compounds produced as by-products of ovarian activity, i.e. as fertility cues [38, 39, 45, 66]	Pro: Changes in ovarian activation cause distinct changes in cuticular hydrocarbon profiles in both social insects [11, 38, 39, 63] and solitary arthropods (e.g. the house fly [115–117], fruit flies [118, 119], cockroaches [120], burying beetles [121], and spiders [122]; reviewed in [45]), and some of these fertility-linked hydrocarbons are similar in structure to known hydrocarbon queen pheromones [9–11].
The production of fertility-linked compounds is under endocrine control	Pro: Production of fertility-linked hydrocarbons is under juvenile hormone control in flies [119], cockroaches [120], burying beetles [123], ants [66, 86], and wasps [124, 125] and under ecdysteroid control in the housefly [117].
Fertility cues can be emitted even if they reduce the fitness of the individuals producing them	Pro: In both ants [40, 41] and honeybees [67], reproductive workers produce specific hydrocarbons that are used by other workers to recognise and police them.
2. Queen pheromones are derived from female sex pheromones used in mate attraction or courtship in solitary ancestors [13]	Pro: Sex pheromones, like queen pheromones, are produced just before mating, before egg-laying commences.
	Contra: Unlike queen pheromone signals, female sex pheromone signals are directed at males rather than females, and the signal does not inhibit reproduction.
Social insect queen pheromones are structurally similar to pheromones used to attract mates or induce male copulation in solitary ancestors.	Pro: Compounds similar in structure to known hydrocarbon queen pheromones or fertility-signalling pheromones [9–11, 40, 41] are used in mate recognition or as contact sex pheromones in many solitary insects (e.g. 3-methylheptacosane in the parasitic wasp *Lariophagus distinguendus* [126], 3-methylnonacosane and 3-methylhentriacontane in the parasitic wasp *Dibrachys cavus* [127], and long-chain alkanes in *Nasonia vitripennis* [128], alkenes in the solitary bees *Megachile rotundata* [71], *Osmia rufa* [62], *Colletes cunicularius* [129], alkanes and alkenes together in *Andrena nigroaenea* [130], and housefly *Musca domestica* [131] and various linear alkanes, methyl-branched alkanes and alkenes in beetles [60]).
Social insect queen pheromones retain their primary function in mate attraction or courtship.	Pro: Honeybee QMP serves a dual role as a sex pheromone and as a sterility-inducing queen signal [4].
	Pro: Limited evidence that some cuticular hydrocarbons are used as contact sex pheromones in some ants and primitively eusocial Halictid bees [62].
3. Queen pheromones are derived from oviposition deterring pheromones that were initially used to regulate the reproduction of fertile females [13].	Pro: Females of many solitary insect reduce their egg-laying in response to pheromonal cues (oviposition deterring pheromones) left by conspecifics [72].
	Pro: Oviposition inhibiting pheromones reduce egg-laying by other females [72], as is the case for social insect queen pheromones.
Social insect queen pheromones are structurally similar to oviposition deterring pheromones in solitary ancestors.	Pro: The oviposition deterring pheromones of the parasitoid wasp *Eupelmus vuilleti* include several long-chain linear alkanes and 3-methyl-branched monomethyl alkanes [73] that are identical or structurally similar to known queen pheromones [9–11].
	Contra: Oviposition inhibiting pheromones in other insect species can have very different chemical structures [72], with no chemical similarity to known queen pheromones.

Unfortunately, as yet, no ODPs that would help to regulate reproduction or oviposition behavior have been documented in any primitively eusocial insects. Nevertheless, it does seem likely that hydrocarbon signals left inside cells or on eggs could well serve this purpose [74].

Are queen pheromones agents of control or mere signals?

Aside from the issue of how queen pheromones first evolved, another important ongoing debate is whether queen pheromones are best considered as honest signals for the presence of a queen ("honest signal hypothesis") [39, 63, 75, 76], or rather as manipulative agents that chemically suppress workers against their own reproductive interests ("queen control hypothesis") [21, 77–80] (Fig. 3, Table 2). Several recent reviews have addressed this issue [14, 15] but failed to obtain unambiguous support for either theory. On the basis of our discovery of conserved fertility-linked hydrocarbon queen pheromones [9–11] and some recently published comparative datasets [17–19] we will here argue, however, that the queen signal hypothesis clearly is best supported. The older of the two theories – queen control – suggests that the queen's pheromonal blend chemically sterilizes workers, thereby

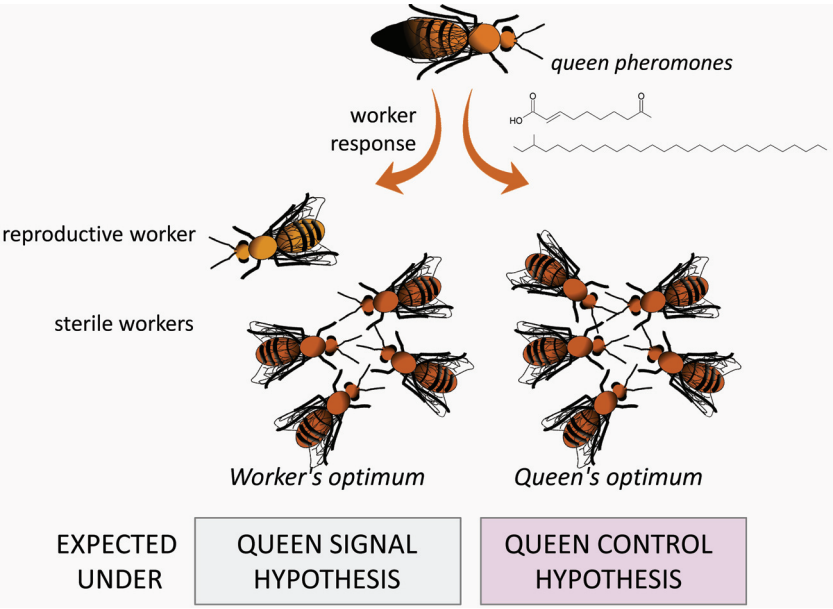

Figure 3. Two hypotheses on how queen pheromones might function: as honest signals or as manipulative agents of control. According to the queen signal hypothesis (bottom left) [39, 75, 76], workers respond to the queen's pheromones to the extent that it serves their own evolutionary interests. This implies the presence of an optimal or "evolutionarily stable" fraction of workers that reproduce inside the colony [17–19, 81, 83, 84]. That is, although most of the workers would be selected to respect the queen signal by remaining sterile, some would benefit from cheating by laying their own eggs (worker shown in yellow). An evolutionarily stable level of worker reproduction is reached when the direct fitness benefits, linked with the workers' own direct reproduction, balance with the indirect fitness costs incurred to the rest of the colony [17–19, 81, 83, 84]. By contrast, the queen control hypothesis (bottom right) [21, 77–80] posits that the queen's pheromones chemically manipulate the workers to remain sterile, against the workers' own reproductive interests. In this case, the workers are prevented from laying eggs, even if some would benefit from direct reproduction.

manipulating them to remain sterile against their own reproductive interests [21, 77–80]. By contrast, the honest signal hypothesis posits that in the presence of a fecund queen, workers would be best served if they did not reproduce and instead focused on the rearing of genetically related siblings [75, 76]. Given that such a response would also help the queen to retain her reproductive monopoly, this would lead to strong selection on queens to produce a reliable or "honest" signal indicating her presence. Theoretical models presented various scenarios in which workers collectively benefit most by not reproducing in the presence of the queen, or where they would benefit from inhibiting or "policing" the reproduction of other workers ([19, 81, 82], reviewed in [83]). This is surprising, given that in these social Hymenopteran insects, workers in principle are capable of reproducing by laying unfertilized, male eggs. In species such as the honeybee, however, where the queen mates multiple times, workers are collectively more related to the sons of the queen (brothers, related by 1/4) than to the sons of other workers (mostly half-nephews, related by only 1/8) [84, 85]. In such species, this favors the workers to police each other, either by cannibalizing each other's eggs or by aggressing the transgressing workers [83–85], which in turn reduces the incentive for workers to try to reproduce in the first place [17, 18, 76, 81, 85]. In addition, even in species where queens are single-mated and workers are thus full siblings, models have demonstrated that workers can benefit genetically from policing worker reproduction or from deliberately giving up all reproduction [19, 82, 83], for example when high levels of worker reproduction would compromise the functioning of the colony or lead to an overproduction of males [19, 82].

In support of the honest signal hypothesis, studies have shown that levels of worker reproduction in different species of social insects are in line with the workers' own genetic interests [17–19]. For example, in a comparative study of nine species of wasps and the honeybee, it was found that in species with queen polyandry and low sib–sib relatedness, very few workers attempt to reproduce in the presence of the queen because of the presence of highly efficient policing, whereas in species with single mating and high relatedness, any queen signals are largely ignored and many workers try to reproduce in presence of the queen (Fig. 4A, [17]). In queenless colonies, however, the relationship is reversed, resulting in increased worker reproduction in species with low relatedness, due to the absence of policing under such conditions and the competition among distantly related kin (Fig. 4A, [81]). These observations fit with workers respecting the queen's signals to the extent that these signals further their own fitness interests [18]. Similarly, in a comparative study of eight species of stingless bees, which were all monandrous, levels of worker reproduction were in agreement with the workers' own theoretical evolutionary optimum and correlated with the costs and benefits for them to produce their own sons in the different species examined (Fig. 4B, [19]). All these results would be difficult to explain by the queen control hypothesis, as this theory would not predict any systematic correlation between either colony kin structure or costs and benefits of worker reproduction and the degree to which the queen could manipulate workers to remain sterile [18].

Another piece of evidence that supports the honest signal hypothesis is that odour profiles correlate very accurately with fertility in different species of ants and wasps (e.g. [18, 38, 39, 63, 86]). In addition, reproductive workers often appear to have chemical profiles that are similar to the queen's bouquet, even if this causes them to be easily detected and attacked by police workers (e.g. [18, 40]). This suggest that their specific odour profiles are uncheatable "indices of fertility" [14, 66]. The exact gene regulatory and endocrine pathways that would explain this association still remain to be elucidated, but several studies indicate that hydrocarbons can be produced as incidental by-products of ovarian activation, and can be under control of the same hormones that regulate oogenesis (cf. Table 1, "ovarian by-product hypothesis"). Against this

Table 2. Predictions of two opposing hypotheses regarding the way in which social insect queen pheromones could function, namely as honest signals for the presence of a fertile queen (queen signal hypothesis) or as agents of control that chemically sterilize the workers against their own reproductive interests (queen control hypothesis)

Predictions		
Queen signal hypothesis: Workers respond to the queen signal by remaining sterile provided that this serves their own inclusive fitness interests [39, 75, 76]	**Queen control hypothesis: Workers are manipulated to remain sterile against their own reproductive interests [21, 77–80]**	**Best empirical support for**
The degree to which the workers should respect queen signals depends on the colony kin structure, because that affects how well queen and worker fitness interests are aligned with one another (Fig. 4) [17, 18, 76, 81].	The degree to which the workers should respect queen signals should not systematically depend on colony kin structure, because the manipulative power of queen pheromone should not be connected to colony kin structure [18].	Queen signal hypothesis, since there is good evidence from many species of social insects that levels of worker reproduction in presence of the queen are in line with the workers' own best interests (cf. Fig. 4) [17–19, 76, 81, 83, 84].
Queen pheromone production should be strongly correlated with queen fecundity and should be honest signals of fertility [75].	Queen pheromone production should be adjusted to colony size [75], but not necessarily queen fecundity, although in many cases both are expected to be highly correlated.	Queen signal hypothesis in the case of hydrocarbon queen pheromones [9–11], since honest cuticular hydrocarbon signals of fertility are found in many ants, bees, and wasps [38, 39, 63]. However, hydrocarbon fertility signals correlate better with colony size than egg-laying rate in the ant *Camponotus floridanus* [87], and 9-ODA and QMP production in the honeybee has been claimed to be not strictly correlated with queen fertility [80], although they do appear to be strongly influenced by insemination volume and mating status [88, 89] and workers have been shown to be more attracted to QMP extracts of fertile queens [91].
There is evolutionary conservation of queen pheromones due to balancing selection on efficient communication of fertility [16].	Queen pheromones experience fast and diversifying selection due to an ongoing arms race between the queen and workers [16].	Conserved composition and bioactivity of social insect pheromones among different species supports the queen signal hypothesis [14]: in honeybees, QMP compositions are similar and bioactive across related species [21, 22, 92, 96, 97], queen pheromones are similar across genera and inhibit worker reproduction across different *Temnothorax* and *Lasius* ants [10, 93] and Vespine wasps [18] and structurally identical or related cuticular hydrocarbons inhibit worker reproduction in wasps, bumblebees and ants [11].
Queen pheromones are complex blends of compounds to increase the reliability and accuracy of the fertility signal [15] or to balance different functions [37] or due to conflicts between individual and collective worker interests that favor some workers to mimic the queen signal, thereby requiring complex blends to maintain signal honesty.	Queen pheromones are complex blends of compounds because of an ongoing evolutionary arms race between queens and workers [68, 75, 79].	Inconclusive.
Queen pheromones primarily affect the sensory system of workers, i.e. through olfaction or gustation, because they should merely constitute a signal [102], although exploitation of pre-existing endocrine or neurological systems is also possible.	Queen pheromones are ingested or absorbed through the cuticle and act like a hormone to stop the workers from reproducing against their own best interests [102].	Queen signal hypothesis in the case of hydrocarbon queen pheromones [9-11], as they are sensed by olfactory receptors [46, 59, 132] and do not have hormonal effects.

(*Continued*)

Table 2. (*Continued*)

Predictions		
Queen signal hypothesis: Workers respond to the queen signal by remaining sterile provided that this serves their own inclusive fitness interests [39, 75, 76]	**Queen control hypothesis: Workers are manipulated to remain sterile against their own reproductive interests [21, 77–80]**	**Best empirical support for**
		Possible evidence for queen control in the case of HVA in honeybees, which has a structural similarity to dopamine and exploits a neuromodulatory system in worker bees that regulates ovary activation [104, 105, 133], although a direct inhibitory effect on worker ovary development has not yet been demonstrated. For honeybee 9-ODA, there is evidence both for direct olfactory reception [26] as well as activity as a hormone, since 9-ODA inhibits ovary development when administered to fruit flies [106] and causes partial inhibition of worker ovary development when injected [21]. Exploitation of pre-existing endocrine or neurological systems, however, is also possible under the queen signal hypothesis.
There may be a cost associated with the production of queen pheromones, as that would help to keep the signal honest [66, 74].	There may be a cost associated to the production of queen pheromones, because queens are inhibited by the pheromone themselves.	Inconclusive.
Workers that happen to interact less with the queen should reproduce more, as they have less accurate information available about the queen's presence.	Workers are selected to avoid interacting with the queen in order to escape queen control [107].	Inconclusive.
Worker ovaries regress when their mother queen is returned, because queen presence is signaled and fitness interests are aligned [81, 83, 84].	Worker ovaries regress when their mother queen is returned, because queen pheromone directly inhibits ovary development [108, 109].	Inconclusive.
Worker reproduction is negatively correlated with the number of queens present, because lower relatedness selects for more worker policing [83, 84].	Worker reproduction is negatively correlated with the number of queens present, because more queens produce more pheromone [75].	Inconclusive.

theory, it has been suggested that hydrocarbon fertility signals correlate more with colony size than with queen fertility in the ant *Camponotus floridanus* [87], even if both would be expected to be highly correlated with each other. In addition, 9-ODA and QMP production in the honeybee has been claimed to be not strictly correlated with queen fertility [80]. Later studies, however, have demonstrated that QMP production and composition are influenced by insemination volume and mating status [88–90], and queens inseminated by multiple males and more fecund queens produce a QMP blend that is more attractive to workers [89, 91]. These data are actually consistent with the queen signal hypothesis and with QMP composition reliably signalling the queen's reproductive quality to the workers [89–91].

Further support for the honest signal hypothesis comes from the degree of conservation of queen and fertility signals in different groups of social insects (e.g. [10, 11, 14, 16, 18, 92, 93]). Under the queen control hypothesis, queen pheromones would be predicted to evolve quickly, because of the evolutionary instability of manipulative queen control [16]. That is, if the queen manipulated workers to remain sterile against their own reproductive interests, it would be expected that workers should quickly evolve tolerance against the queen's pheromones and reproduce nonetheless. The queen, in turn, would then be expected to start producing higher quantities or novel compounds to remain in control, thus leading to an evolutionary arms race between the queen and workers [16, 94, 95]. Hence, the queen control hypothesis would predict that queen pheromones evolve more quickly and should be composed of more complex blends than under a queen signal scenario [10, 15, 16] (Table 2). Comparative evidence, however, provides no evidence for rapid evolution of social insect queen pheromones [10, 75, 93]. For example, in honeybees, QMP composition appears to be similar among related species [92, 96], and 9-ODA acts as the principal sterility-inducing queen pheromone in both *A. mellifera* and *A. cerana* (Fig. 1A, B, [21, 22]). Furthermore, queens are able to inhibit worker reproduction even in artificial mixed-species

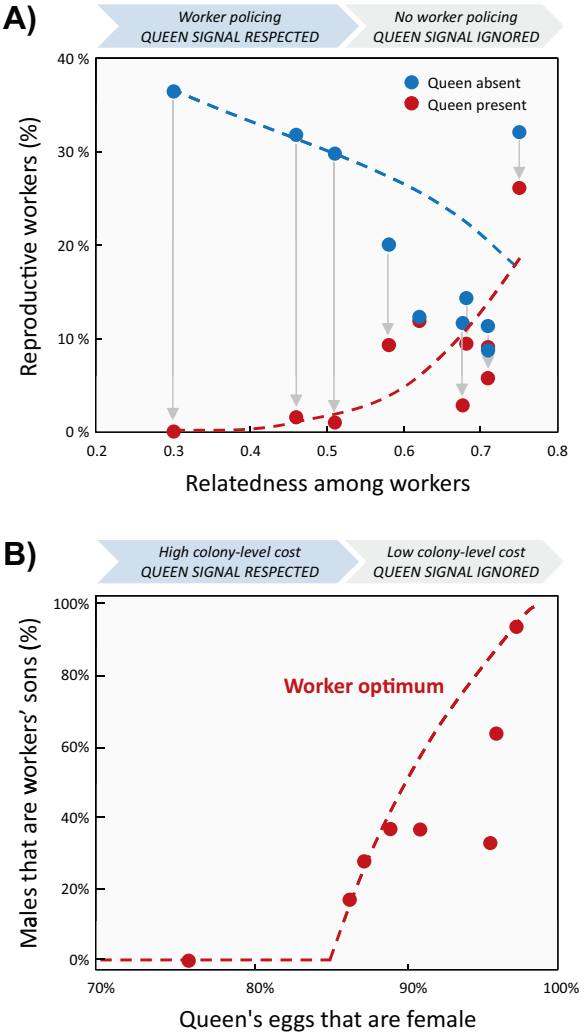

Figure 4. Queen pheromones as honest signals: comparative support. **A:** A comparative analysis of nine wasp species and the honeybee shows that only in species with low genetic relatedness, caused by queen polyandry, workers strongly respect the queen's pheromonal signals, as can be seen from the fact that only in those species, far fewer workers try to reproduce in the presence of the queen (red points, cf. arrows) [17, 18, 83]. Data from Wenseleers and Ratnieks [17]. **B:** A comparative analysis of eight *Melipona* stingless bee species shows that workers do not respect the queen's pheromonal signals and that many workers reproduce in species where the mother queen lays many female eggs. This is because in those species, the workers can afford to replace some of the female worker-destined eggs by their own sons. By contrast, in species where there are few female eggs available (<ca. 85%), workers are better off respecting the queen signal, in order to avoid costs to the growth of the colony caused by a shortage of workers. The dashed line represents the theoretical evolutionary optimum level of worker reproduction, calculated from the workers' perspective. Data from Wenseleers et al. [19].

colonies [97]. Similarly, hydrocarbon queen pheromones were similar across different Vespine wasps [18] and *Temnothorax* and *Lasius* ants [10, 93], and also show cross-activity when tested in related species [10, 93]. In formal phylogenetic analyses, queen pheromones also displayed a high degree of evolutionary conservation [11]. These data suggest that the specific compounds used as queen pheromones have been quite stable over evolutionary time, and are not subject to antagonistic co-evolution between queens and workers. Stabilizing selection caused by a role in desiccation resistance coupled with a low cost of production, however, could also play a role in this evolutionary conservation.

The complexity of honeybee QMP pheromone blend [79, 98] and the fact that egg-laying honeybee workers appear to mimic the queen's pheromone bouquet [68, 69] has occasionally been cited as possible evidence for queen control. Similarly complex blends, however, could also evolve under the queen signal hypothesis as a way for the queen to increase the reliability and accuracy of the fertility signal [15]. In addition, combined use of pheromones of different degrees of volatility may be required to serve the different functions of social insect queen pheromones [37]. For example, in the honeybee, inducing queen retinue behavior (attracting workers) or attracting drones during a mating flight, would require volatile compounds like QMP [23], whereas signalling queen fertility or dominance may be more reliable via the use of non-volatile pheromones [37]. Finally, conflicts between individual and collective worker interests could favor some workers to mimic the queen signal, and result in complex blends in order to maintain signal honesty. The case of anarchistic and parasitic Cape honeybee, which workers lay eggs that evade policing presumably by chemically mimicking those of the queen [99–101] support this possibility.

Although overall the collective evidence clearly provides the best support for queen pheromones merely acting as honest signals, it has been suggested that queen control could still occur as part of a queen-worker arms race in which the queen is temporarily ahead of the workers [95, 102]. For example, the queen could manipulate the reproduction of workers via the release of specific hormones or toxins. This possibility has been rekindled by the documentation of such a manipulation in the context of intersexual conflict in the fruit fly [102]. Here, males transfer over 100 proteins during mating, causing a wide variety of fitness related effects in females, including a decreased sexual receptivity, which apparently reduces the females' own fitness [103]. In fact, one of the compounds of the honeybee QMP, homovanilyl alcohol (HVA) has a structural similarity to dopamine, and was suggested to exploit a pre-existing neuromodulatory system in worker bees that regulates ovary activation [104, 105]. As yet, however, the effect of individually administered HVA on honeybee worker ovary inhibition remains untested. In addition, for the main compound of honeybee QMP, 9-ODA, there is evidence both for direct olfactory reception in the antennae [26] as well as for activity as a hormone, since 9-ODA has been shown to inhibit ovary development when administered to fruit flies [106]. Furthermore, it also causes partial inhibition of worker ovary development when directly injected into honeybee workers [21]. Although these results demonstrate that honeybee queen pheromones likely exploit some pre-existing physiological systems [14], they do not necessarily imply that the workers' reproduction is suppressed against their own reproductive interests. Several other, similarly ambiguous predictions have been made, for example with respect to the cost of producing queen pheromones ([66, 74], cf. section

below), worker avoidance of queen control [107] or the reversibility of the effect of queen pheromones on workers' reproduction [108, 109] (Table 2). None of these predictions is falsifiable, as the empirical data can all potentially be explained by either queen control or queen signalling. Nevertheless, it is clear that overall, based on the other evidence we cited, the queen signal hypothesis is best supported.

How is signal honesty maintained?

If queen pheromones function as honest signals instead of manipulative chemicals, the question arises as to what keeps the signal honest [66, 70]. A first possibility would be that there is a classic Zahavian cost associated with the production of the pheromone ([9, 15, 16, 66, 74], see also Table 2). For example, the production could be physiologically costly or the pheromone could be somewhat toxic, so that only individuals of high quality (i.e. fertile queens) would be able to afford to produce the signal and obtain fitness benefits from the signalling system ("the handicap principle") [9, 15, 16, 66, 74], e.g. through greater suppression of workers' reproduction or to avoid being killed by the workers if they perceive the queen to be of insufficient quality [110]. Alternatively, it is possible that honesty would be maintained as a result of the queen pheromone being an uncheatable index of queen quality or queen fertility [66, 70]. This could be the case if queen pheromones are intrinsically linked to ovary development in such a way that the signal simply cannot be faked ([70], "index hypothesis"). As cited above, for hydrocarbon queen pheromones there is evidence to support this idea (cf. Table 1, "ovarian by-product hypothesis"). A third possibility, which is sometimes overlooked, is that signal honesty may simply be maintained by the genetic relatedness and the alignment of fitness interests between queens and workers (e.g. [111]). That is, if the queen benefits from producing a queen signal and workers benefit from respecting the signal, then the common interests of the signaller queen and receiver workers will ensure that the signal is kept reliable without requiring queen pheromone production to be costly [70, 111]. The data illustrated in Fig. 4 demonstrate that relatedness and indirect genetic benefits can indeed result in the partial or even complete alignment of queen and worker interests, and select workers to respect queen's pheromone signals to varying degrees.

Conclusions and outlook

The discovery of conserved queen pheromones in social insects has prompted many new questions with regard to how these important signalling molecules first originated and how they were later elaborated. Although some of the theories and hypotheses we have suggested, such as the queen signal hypothesis, are already well-supported, several others still require further testing. Information on the rate of evolution of social insect queen pheromones and similarities to compounds used in other contexts in solitary insects, for example, should still be tested at the biosynthetic and receptor level. These lines of research still require more detailed phylogenetic analyses (cf. [112, 113]) as well as the study of closely related social species and solitary outgroups and of fertility signals and mate recognition pheromones in primitively eusocial species. In addition to bioassays, such studies should ideally use highly standardized chemical analyses to avoid any technical biases, e.g. through the use of modern metabolomics pipelines (e.g. [114]) and mass spectrometry methods to enable detailed characterization both of volatile and non-volatile signalling molecules. They way in which caste-linked hydrocarbon profiles are detected and integrated in the brain also clearly requires further study [46]. Future studies can be aimed towards resolving some of these outstanding questions, and thereby help us to gain novel insights into both the proximate and ultimate, evolutionary aspects of a key signalling system in insects.

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